Spring 2018

Detection of Borrelia burgdorferi in historic tick samples and its relevance to the white-tailed deer population in New Hampshire

Tasya Rakasiwi
University of New Hampshire, Durham

Detection of *Borrelia burgdorferi* in historic tick samples and its relevance to the white-tailed deer population in New Hampshire

TASYA RAKASIWI
DEPARTMENT OF MOLECULAR, CELLULAR, AND BIOMEDICAL SCIENCES
University of New Hampshire Honors Senior Thesis

TABLE OF CONTENTS

ACKNOWLEDGEMENTS

Brian Stevens, D.V.M., D.V.Sc.

Christopher Benton, Ph.D.

Alan Eaton, Ph.D.

Denise Bolton, B.S.

Feng Xu, Ph.D.

Daniel Bergeron

State of New Hampshire Public Health Laboratories

U.S. Department of Agriculture

ABSTRACT

The cases of Lyme disease in New Hampshire have increased over time. There are speculations that increasing number of Lyme disease cases in New Hampshire are due to environmental factors, such as warmer climate, white-footed mouse population, white-tailed deer population, opossum population, and forestation coverage. In this study, we processed whole tick samples from 2000, 2001, and 2003 for *Borrelia burgdorferi* by Real-Time TaqMan PCR. In addition, we also processed homogenized tick samples from 2010, that previously tested positive for *B. burgdorferi* and had been stored at -80°C since 2010, for repeat *B. burgdorferi* testing by Real-Time TaqMan PCR. Then, the number of reported positive tick samples from the years 2000, 2001, 2003, 2009, 2010, and 2011 were correlated with white-tailed deer population and Lyme disease cases. Based on our analysis, there was an indirect relationship noted between white-tailed deer population, which is highly suggestive of the relationship between host diversity and Lyme disease cases. On the other hand, the rate of positive tick samples exhibited similar trend as Lyme disease cases. Due to poor staffing and funding issues, the NH Department of Human and Health Services were not able to obtain any tick samples in 2002, from 2004 to 2008, and from 2012 to 2017. We were unable to correlate Lyme disease cases to neither white-footed mouse population nor opossum population, since the NH Fish and Game do not keep track of these two populations. Of the 141 ticks collected in 2000, 2001, and 2003, 44 ticks tested positive for *B. burgdorferi*. These sample became the oldest, documented tick samples in the state of New Hampshire, which tested positive for *B. burgdorferi*. In addition, there were insignificant deviations noted between Ct values of the 2010 samples, which were processed in 2010 and 2018. Therefore, the unremarkable difference in Ct values suggest that cryopreservation seems to be the most optimal method of preserving DNA. It was also noted in

this study, historic samples had significantly lower DNA concentration than the 2010 samples. We attributed the significant difference to time of storage and method of DNA preservation. We attempted to sequence tick samples for Next Generation Sequencing. DNA of tick samples from 2000, 2001, 2003, and 2010 were quantified in Qubit Fluorometer. However, DNA concentration of individual tick samples were insufficient for prokaryotic enrichment, thus the DNA from positive tick samples in 2010 were pooled together. The pooled DNA was reprecipitated and quantified, but the DNA concentration was still insufficient to proceed with enrichment and sequencing.

INTRODUCTION

The New Hampshire Department of Humanities and Health Service reported that cases of tickborne disease have significantly increased over the past twenty years. In New Hampshire, black-legged tick, or *Ixodes scapularis*, is the most common vector for *Borrelia burgdorferi* (Lyme disease), *Anaplasma phagocyphilum* (Anaplasmosis), *Babesia sp.* (Babesiosis), and Powassan virus. The most common reservoir for all four pathogens in New Hampshire is the white-footed mouse (Division of Public Health Services 2016). Understanding the *I. scapularis* life cycle, pathogen, and reservoir hosts are impertinent for the transmission of tickborne diseases (Division of Public Health Services 2015). *I. scapularis* hatch from eggs as larvae in the summer, during which they have the opportunity to obtain their first blood meal from small mammals or birds. The first bloodmeal is the first opportunity for the tick to be infected by a pathogen (Division of Public Health Services, 2015). After engorgement from the first blood meal and detachment from host, the larva molts into a nymph. The nymph searches for its second blood meal from either small mammals, birds, or humans during the following spring or summer. After the completion of the second bloodmeal and detachment from host, the nymph molts into an adult tick in the fall or following spring. Adult tick continues to be active until the temperature in its environment is below 41 degrees Fahrenheit or its environment has become covered with snow (Division of Public Health Services, 2015). Typically, adult tick will feed on medium or large animals, such as white-tailed deer, to be used as a reproductive host. The adult tick will lay its eggs in spring. Due to multiple blood meals, and thus multiple chances of infections, infection rate of an adult tick is typically higher than other stages of a tick lifecycle (Division of Public Health Services, 2015). Despite a lower infection rate, a nymph has the highest likelihood of transmission in a tick lifecycle. This is due to a nymph's miniscule size,

which makes it harder to detect than an adult tick, thus providing the nymph with a longer period of attachment to host. This longer period of attachment to host subsequently increases the likelihood of transmission of pathogen to a naïve and an uninfected host (Division of Public Health Services, 2015). Although white-tailed deer are quintessential hosts to sustain the tick population (Department of Human and Health Services 2015;Werden, et al. 2014), however, the white-tailed deers are poor reservoirs for *B. burdorferi*, because of their ability to naturally form antibodies against *B. burgdorferi* (Magnarelli et al.1993).

The environment plays an important role in the transmission of tickborne diseases. For example, climate change has been shown to play a critical role in tick host population and tick development (Martin 2010; Werden, et al. 2014). Due to climate change, winter has become warmer and shorter than before, which has subsequently led to an increase in hosts surviving through the winter, therefore providing abundant food sources for larvae and nymphs to survive. Another example is significant forestry coverage in New Hampshire, which correlates to production of seeds and nuts—primary food sources of reservoir and reproductive hosts of ticks, such as white-tailed deer (Division of Public Health Services 2015). Due to the environment's major role in the transmission of tickborne diseases, there have been attempts of using environmental factors to monitor or prevent tickborne diseases. For example, previous studies have suggested the feasibility of monitoring white-tailed deer population and temperature as a predictor for emerging tickborne diseases, (Wilson et al. 1988; Bouchard et al. 2013). Because of the strong correlation between deer and tick populations, many communities have implemented deer reduction programs in the past. For example, Great Island peninsula in Cape Cod, MA reduced the deer population by approximately 97% from 1982 to 1984 (Wilson et al. 1988). Three years after the reduction program, there is noted decrease in larva and nymph population

and reported tickborne disease cases. Another example of the success of these reduction programs is when Ipswich, MA implemented a deer reduction program over the course of seven years (Deblinger et al. 1993). Following the deer reduction, the study reports 50% decrease of larva population and 41% nymph population (Deblinger et al. 1993). Although there have been multiple studies to further prove the correlation between tick and deer populations, but the correlation between deer population and Lyme disease cases has not been evaluated (Kugeler et al. 2015).

In this study, we examined the population of both white-tailed deer for any potential correlation with the rate of positive tick tests. We did not correlate Lyme disease cases with white-footed mouse population, since the white-footed mouse population are not tracked. We attempted to compare the rate of positive tick tests with forestry coverage, but we were unable to obtain any forestry data prior to the year of 2006. Due to insufficient data, we were unable to determine any significant finding when comparing the forestry data and rate of positive tick samples. We did, however, compare the rate of positive tick tests and number of reported Lyme disease cases. We also analyzed tick samples collected by Dr. Alan Eaton, an entomologist at the University of New Hampshire, and Maine Medical Center, contracted employee for the state of New Hampshire in 2010, for the presence of *Borrelia burgdorferi*. The tick samples were not analyzed for the presences of *Anaplasma phagocyphilum*, *Babesiosis sp.*, and Powassan virus due to the low incidence rates in NH. Within the years of 2010 and 2015, there were approximately 30 to 150 cases of Anaplasmosis, 10 to 80 cases of Babesiosis, and 1 case of Powassan Virus (Division of Public Health Services 2016). Consequently, samples were only tested for the detection of *Borellia burgdorferi*, the spirochete that causes Lyme disease, due to its high incidence rate in the state of New Hampshire. As reported by NH Department of Health

and Humanities, the incidence rate of Lyme disease in NH has remained high over the 1,000s per capita since 2008; in fact, NH had the highest incidence rates in the nation for the years 2008 and 2012. In this study, ethanol-preserved, whole tick samples from 2000, 2001, and 2003 were processed through Real-Time TaqMan PCR to detect *B. burgdorferi*, as well as previously positive, cryopreserved, and homogenized tick samples from 2010. The target site of amplification was the highly-conserved portion of the flagellin gene, which yielded DNA fragments of 276 bp long (Picken 1992; Zeidner et al. 2001). DNA from each sample was quantified by Qubit Fluorometer. However, due to poor DNA concentration, tick samples were pooled, reprecipitated, then re-quantified. The DNA concentration of the pooled samples remained low, thus tick samples could not undergo prokaryotic enrichment process. Consequently, tick samples were not sequenced in this study.

RESULTS

Borrelia burgdorferi Detection

141 tick samples were collected from Strafford, Rockingham, and Merrimack counties as shown in Table 1. The Ct value is determined by when the hyperbolic curve passes the threshold, which is shown in Figures 1, 2, and 3. A sample was considered positive for *Borrelia burgdorferi* if its Ct value is less than 38. Out of 141 samples, 43 tick samples were positive for *Borellia burgdorferi*. Both samples 29 and 130 had Ct values of 38, thus their amplification curve were analyzed. The amplification curve of sample 29 was hyperbolic and appears similar to the amplification curve of positive control, thus sample 29 was deemed positive for *Borellia burgdorferi* (Figure 3) The amplification curve of sample 130 was neither hyperbolic nor resembles the curve of the positive control, thus sample 130 was deemed negative for *Borellia burgdorferi* (Figure 3).

Table 1 This table details the time, host, engorged, location, and Ct value of each tick sample. The tick sample was deemed positive for *Borellia burgdorferi* if the Ct value is less than 38; these samples were highlighted in green. If Ct value is 38, then the sample is deemed positive if the amplification curve is hyperbolic. Ct values greater than 38 were deemed negative for *Borellia burgdorferi*; these samples were highlighted in red. Flagged ticks were collected by waving a cotton flag over higher vegetation.

Sample Number	Date	Host	Engorged?	Location	Ct
1	11/2000	Flagged	No	Newington, NH	35.81
2	11/2000	Flagged	No	Newington, NH	Undetermined
3	11/2000	Flagged	No	Newington, NH	Undetermined
4	11/2000	Flagged	No	Newington, NH	Undetermined
5	11/2000	Flagged	No	Newington, NH	Undetermined

6	11/2000	Flagged	No	Newington, NH	Undetermined
7	11/2000	Flagged	No	Newington, NH	Undetermined
8	11/2000	Flagged	No	Newington, NH	Undetermined
9	11/2000	Flagged	No	Newington, NH	Undetermined
10	11/2000	Flagged	No	Newington, NH	Undetermined
11	11/2000	Flagged	No	Stratham, NH	Undetermined
12	11/2000	Flagged	No	Stratham, NH	36.31
13	11/2000	Flagged	No	Stratham, NH	32.67
14	11/2000	Flagged	No	Stratham, NH	33.91
15	11/2000	Flagged	No	Stratham, NH	Undetermined
16	11/2000	Flagged	No	Stratham, NH	34.04
17	11/2000	Flagged	No	Stratham, NH	Undetermined
18	11/2000	Flagged	No	Stratham, NH	Undetermined
19	11/2000	Flagged	No	Stratham, NH	Undetermined
20	11/2000	Flagged	No	Stratham, NH	36.27
21	5/26/2001	Flagged	No	Concord, NH	35.54
22	5/26/2001	Flagged	No	Concord, NH	Undetermined
23	5/26/2001	Flagged	No	Concord, NH	Undetermined
24	5/26/2001	Flagged	No	Concord, NH	Undetermined
25	5/26/2001	Flagged	No	Concord, NH	Undetermined
26	5/26/2001	Flagged	No	Concord, NH	Undetermined
27	5/26/2001	Flagged	No	Concord, NH	Undetermined
28	5/26/2001	Flagged	No	Concord, NH	Undetermined
29	5/26/2001	Flagged	No	Concord, NH	38.09

30	5/26/2001	Flagged	No	Concord, NH	Undetermined
31	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
32	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
33	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
34	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
35	11/2000 to 5/2001	Flagged	No	Lee, NH	34.41
36	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
37	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
38	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
39	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
40	11/2000 to 5/2001	Flagged	No	Lee, NH	Undetermined
41	5/5/2001	Flagged	No	Durham, NH	34
42	5/5/2001	Flagged	No	Durham, NH	35.72
43	5/5/2001	Flagged	No	Durham, NH	34.63
44	5/5/2001	Flagged	No	Durham, NH	33.96
45	5/5/2001	Flagged	No	Durham, NH	Undetermined
46	5/5/2001	Flagged	No	Durham, NH	32.13
47	5/5/2001	Flagged	No	Durham, NH	35.82
48	5/5/2001	Flagged	No	Durham, NH	Undetermined
49	5/5/2001	Flagged	No	Durham, NH	Undetermined
50	5/5/2001	Flagged	No	Durham, NH	42.31
51	5/31/2001	Flagged	No	Madbury, NH	37.39
52	5/31/2001	Flagged	No	Madbury, NH	Undetermined
53	5/31/2001	Flagged	No	Madbury, NH	Undetermined

54	5/31/2001	Flagged	No	Madbury, NH	Undetermined
55	5/31/2001	Flagged	No	Madbury, NH	Undetermined
56	5/31/2001	Flagged	No	Madbury, NH	Undetermined
57	5/31/2001	Flagged	No	Madbury, NH	Undetermined
58	5/31/2001	Flagged	No	Madbury, NH	Undetermined
59	5/31/2001	Flagged	No	Madbury, NH	35.4
60	5/31/2001	Flagged	No	Madbury, NH	Undetermined
61	11/4/2000	Flagged	No	Portsmouth, NH	Undetermined
62	11/4/2000	Flagged	No	Portsmouth, NH	35.42
63	11/4/2000	Flagged	No	Portsmouth, NH	Undetermined
64	11/4/2000	Flagged	No	Portsmouth, NH	Undetermined
65	11/4/2000	Flagged	No	Portsmouth, NH	Undetermined
66	11/4/2000	Flagged	No	Portsmouth, NH	Undetermined
67	11/4/2000	Flagged	No	Portsmouth, NH	Undetermined
68	11/4/2000	Flagged	No	Portsmouth, NH	Undetermined
69	11/4/2000	Flagged	No	Portsmouth, NH	33.23
70	11/4/2000	Flagged	No	Portsmouth, NH	35.2
71	5/2000 to 11/2000	Flagged	No	Rye, NH	31.74
72	5/2000 to 11/2000	Flagged	No	Rye, NH	32.57
73	5/2000 to 11/2000	Flagged	No	Rye, NH	Undetermined
74	5/2000 to 11/2000	Flagged	No	Rye, NH	Undetermined
75	5/2000 to 11/2000	Flagged	No	Rye, NH	Undetermined
76	5/2000 to 11/2000	Flagged	No	Rye, NH	Undetermined
77	5/2000 to 11/2000	Flagged	No	Rye, NH	Undetermined

78	5/2000 to 11/2000	Flagged	No	Rye, NH	36.64
79	5/2000 to 11/2000	Flagged	No	Rye, NH	32.14
80	5/2000 to 11/2000	Flagged	No	Rye, NH	Undetermined
81	10/2000	Dog	No	Durham, NH	Undetermined
82	10/2000	Dog	No	Durham, NH	Undetermined
83	10/2000	Dog	No	Durham, NH	Undetermined
84	10/2000	Dog	No	Durham, NH	Undetermined
85	10/2000	Dog	No	Durham, NH	Undetermined
86	10/2000	Dog	No	Durham, NH	Undetermined
87	10/2000	Dog	No	Durham, NH	Undetermined
88	10/2000	Dog	No	Durham, NH	Undetermined
89	10/2000	Dog	No	Durham, NH	Undetermined
90	5/6/2001	Human	No	Durham, NH	Undetermined
91	5/6/2001	Human	No	Durham, NH	Undetermined
92	5/5/2001	Flagged	No	Durham, NH	33.19
93	5/5/2001	Flagged	No	Durham, NH	30.85
94	5/5/2001	Flagged	No	Durham, NH	36.59
95	5/5/2001	Flagged	No	Durham, NH	34.78
96	5/5/2001	Flagged	No	Durham, NH	33.28
97	5/5/2001	Flagged	No	Durham, NH	35.1
98	4/2000	Dog	No	Amherst, NH	Undetermined
99	4/2000	Dog	No	Amhert, NH	Undetermined
100	6/26/2001	Human	No	Lee, NH	Undetermined
101	6/26/2001	Human	No	Lee, NH	35.27

102	6/26/2001	Human	No	Lee, NH	Undetermined
103	5/2001	Human	No	Nottingham, NH	Undetermined
104	4/2000	Human	Yes	Newmarket, NH	Undetermined
105	9/2000	Human	No	Dover, NH	32.81
106	4/2001	Human	Yes	Amherst, NH	Undetermined
107	11/2000	Human	Yes	Strafford, NH	34.2
108	10/2000	Human	No	Durham, NH	35.03
109	5/2000	Human	No	Durham, NH	32.26
110	6/2000	Human	Yes	Lee, NH	Undetermined
111	4/2000	Human	No	Lee, NH	Undetermined
112	4/2000	Human	No	Lee, NH	Undetermined
113	4/2000	Flagged	No	Lee, NH	Undetermined
114	11/2000	Cat	Yes	Greendland, NH	31.07
115	6/2000	Flagged	No	Lee, NH	Undetermined
116	6/2000	Flagged	No	Lee, NH	Undetermined
117	5/2001	Human	No	Lee, UNH	Undetermined
118	10/2000	Human	No	Durham, NH	Undetermined
119	10/2001	Human	Yes	Madbury, NH	Undetermined
120	11/2000	Horse	Yes	Amherst, NH	Undetermined
121	11/2000	Horse	Yes	Amherst, NH	Undetermined
122	5/2003	Flagged	No	Lee, NH	34.85
123	5/2003	Flagged	No	Lee, NH	Undetermined
124	5/2003	Flagged	No	Lee, NH	Undetermined
125	5/2003	Flagged	No	Lee, NH	Undetermined

126	5/2003	Flagged	No	Lee, NH	36.91
127	5/2003	Flagged	No	Lee, NH	37.14
128	5/2003	Flagged	No	Lee, NH	Undetermined
129	5/2003	Flagged	No	Lee, NH	35.13
130	5/2003	Flagged	No	Lee, NH	Undetermined
131	5/2003	Flagged	No	Lee, NH	40.13
132	5/2003	Flagged	No	Lee, NH	34.59
133	5/2003	Flagged	No	Lee, NH	36.73
134	5/2003	Flagged	No	Lee, NH	Undetermined
135	5/2003	Flagged	No	Lee, NH	Undetermined
136	5/2003	Flagged	No	Lee, NH	Undetermined
137	5/2003	Flagged	No	Lee, NH	37.03
138	5/2003	Flagged	No	Lee, NH	38.39
139	5/2003	Flagged	No	Lee, NH	Undetermined
140	5/2003	Flagged	No	Lee, NH	Undetermined
141	5/2003	Flagged	No	Lee, NH	Undetermined

A total of 10 tick samples, which were acquired in 2010 and known positives, were also processed through Real-Time TaqMan PCR (Table 2). The 10 tick samples were collected in Strafford County and had the lowest Ct values when the samples were processed in 2010. The tick samples were reprocessed in 2017. The Ct values of all ten samples are lower than the positive samples from 2000 to 2003. Of note, the Ct values of all ten samples after reprocessing in 2017 are comparable to the Ct values acquired in 2010.

Table 2 The following samples were acquired in 2010 by Maine Medical Center. These specific samples were collected in Durham, NH. This table lists the Ct values from when the samples were processed in 2010 and 2017. Quantification were not acquired in 2010, since the intention of the sample collection was for Lyme disease facilitation. All ten samples were not quantified, since DNA concentrations of samples 573 and 614, which have the lower Ct values of the ten samples, had less than 0.5 ng per μL.

Sample Number	Ct Value (qPCR in 2010)	Ct Value (qPCR in 2017)	DNA Concentration (ng/μL)	DNA Concentration (ng/μL) with DNA Concentrator Kit	Pooled DNA Concentration (ng/μL)
570	27.74	28.67	N/A	0.236	0.170
573	26.14	26.26	0.359	0.663	
578	26.98	27.21	N/A	0.163	
581	26.57	27.18	N/A	0.173	
583	27.64	30.42	N/A	0.331	
594	25.97	26.61	N/A	0.305	
596	28.08	27.01	N/A	0.110	
598	27.48	27.46	N/A	0.0840	
603	27.20	27.43	N/A	0.0950	
614	27.59	26.22	0.445	0.217	

DNA Quantification

Some of the genomic tick samples were quantified. The following negative samples were randomly selected for quantification: 24, 33, 39, 49, 52, 64, 73, 80, 81, 93, 97, 104, 109. Sample 46 had a concentration of 0.080 ng/μL, Sample 64 had a concentration of 0.069 ng/μL, and sample 97 had a concentration of 0.088 ng/μL. The remaining negative samples' DNA

concentration was too low to be quantified by the Qubit fluorometer. Samples 45 and 109 were positive samples that were quantified by the Qubit fluorometer. DNA concentration of sample 45 was too low to be quantified by the Qubit fluorometer, while sample 109 had a concentration of 0.059 ng/μL. Two samples, which were acquired from 2010, were also quantified: 573 and 614. Sample 573 had a concentration of 0.359 ng/μL and sample 614 had a concentration of 0.445 ng/μL. After reprecipitation, DNA was quantified in all ten samples. The samples had poor yield of DNA. Sample 573 had the highest concentration of 0.663 ng/μL, while sample 598 had the lowest concentration of 0.0840 ng/μL.

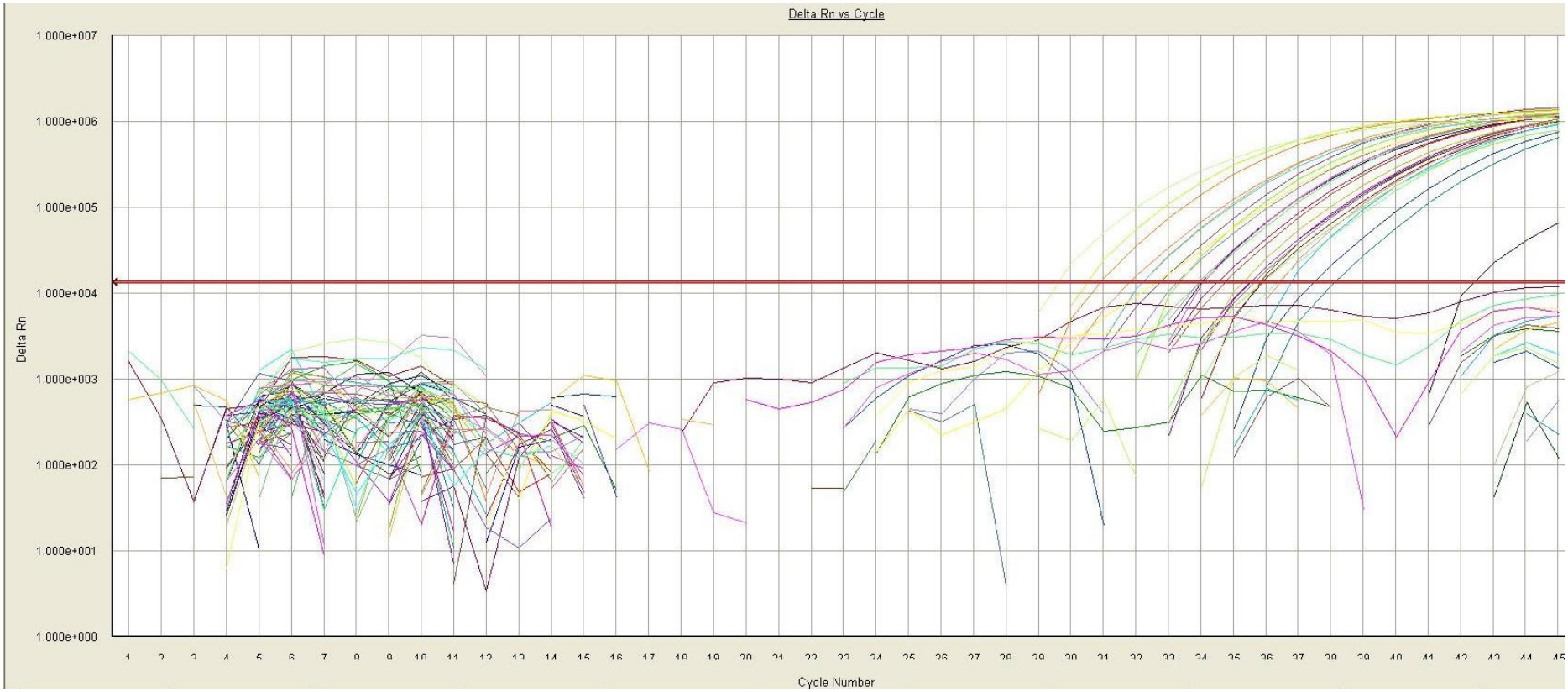

Figure 1 qPCR run of tick samples from 1 to 96. The red horizontal line is the threshold. Samples with Ct lines that did not pass the threshold were negative for the detection of *Borrelia burgdorferi*. Samples with Ct lines that appears parabolic and pass the threshold were positive for the detection of *Borrelia burgdorferi.* The lower the number of cycle it takes for Ct line to pass through the threshold is inversely related to the concentration of *Borrelia burgdorferi* DNA.

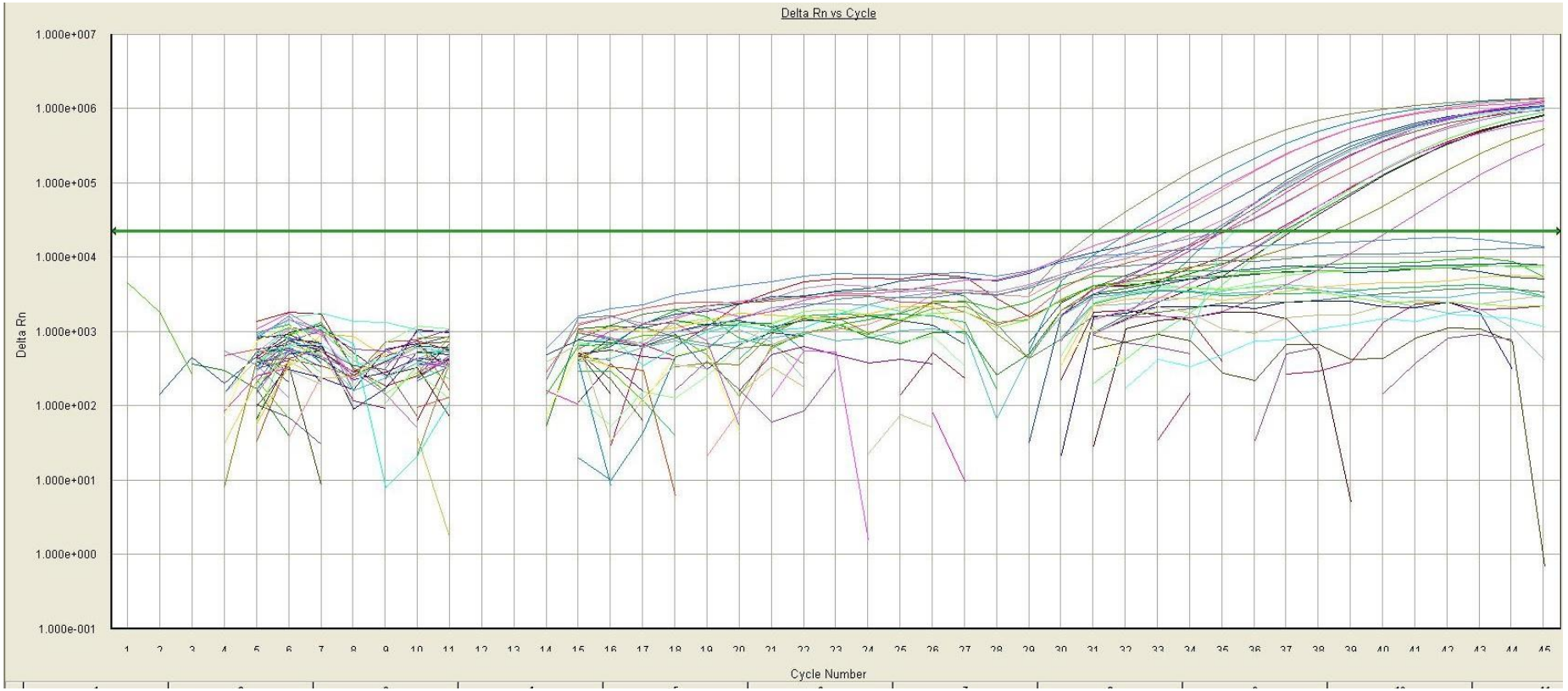

Figure 2 qPCR run of tick samples from 97 to 141. The green horizontal line is the threshold. Samples with Ct lines that did not pass the threshold were negative for the detection of *Borrelia burgdorferi*. Samples with Ct lines that appears parabolic and pass the threshold were positive for the detection of *Borrelia burgdorferi.* The lower the number of cycle it takes for Ct line to pass through the threshold is inversely related to the concentration of *Borrelia burgdorferi* DNA.

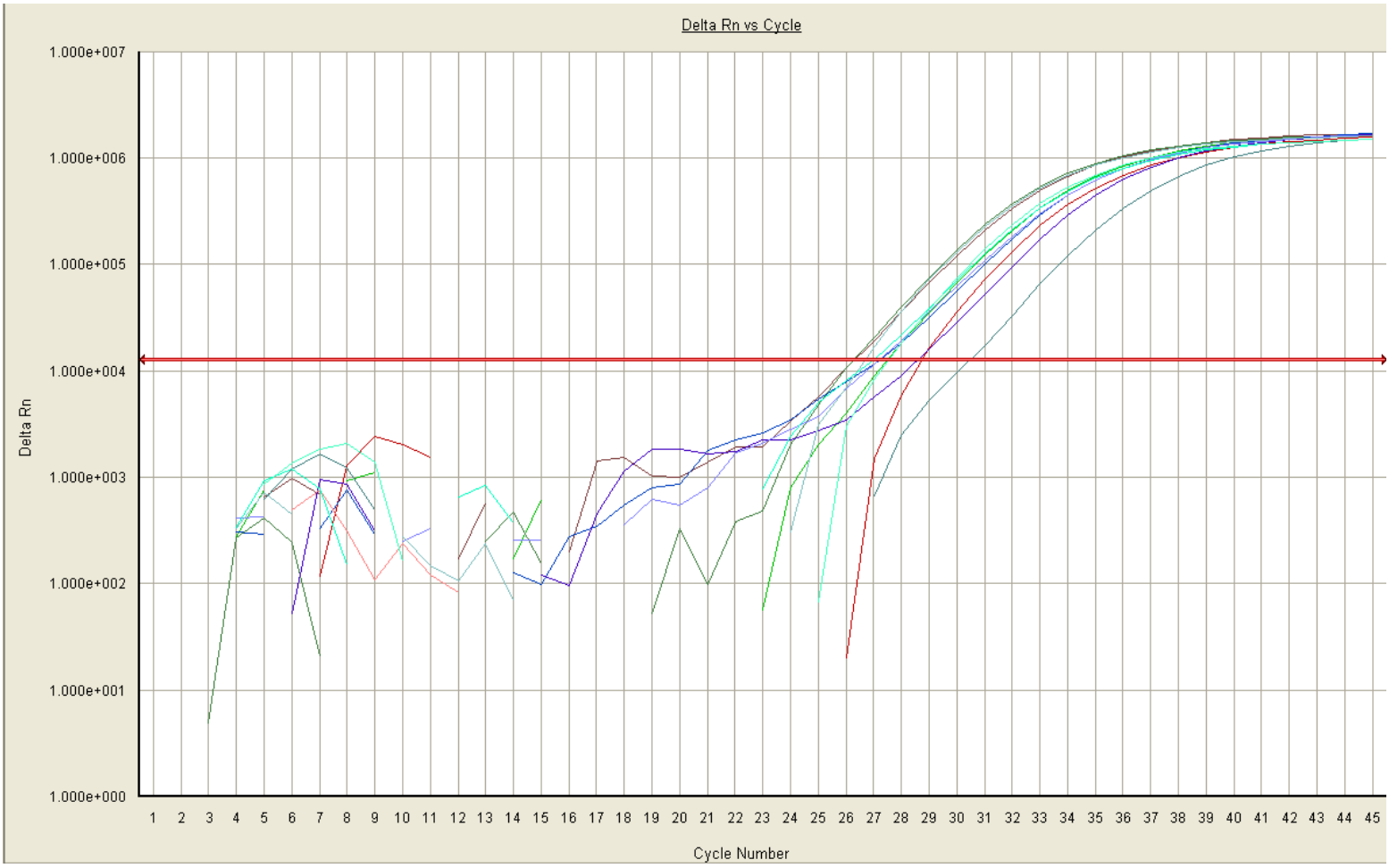

Figure 3 qPCR run of tick samples from 2010. These samples had been processed through qPCR at the time of collection and are known positives. The red horizontal line is the threshold. Since all samples' Ct lines appear parabolic and pass the threshold prior to 38th cycle, they were positive for the detection of *Borrelia burgdorferi.* The lower the number of cycle it takes for Ct line to pass through the threshold is inversely related to the concentration of *Borrelia burgdorferi* DNA.

Environmental Analysis of Positive Tick Sample

Tick samples, that had tested positive for *B. burgdorferi*, in 2000, 2001, 2003, 2009, 2010, and 2011 were compared with deer population and number of reported Lyme disease cases. It is important to note that the processing of tick samples from 2009 and 2011 were previous data collected by the State of New Hampshire Public Health Laboratories. Although a few of the 2010 samples were re-tested in this study, but the 2010 data used for environmental analysis was previously done by the State of New Hampshire Public Health Laboratories. Sample number 71 through 80 were reported to be collected between November 2000 to May 2001 (Table 1). Therefore, the samples collected in the years 2000 and 2001 were grouped together. Due to poor funding and resources, tick testing and collection were not consistently done throughout time. Thus, there are gaps of tick testing data in 2002 and from 2004 to 2008. In the graph comparing positive tick samples and deer population, an inverse relationship may exist between the two data (Figure 4). On the other hand, the graph that compares positive tick samples and Lyme disease cases demonstrated similar, increasing trend over time (Figure 5

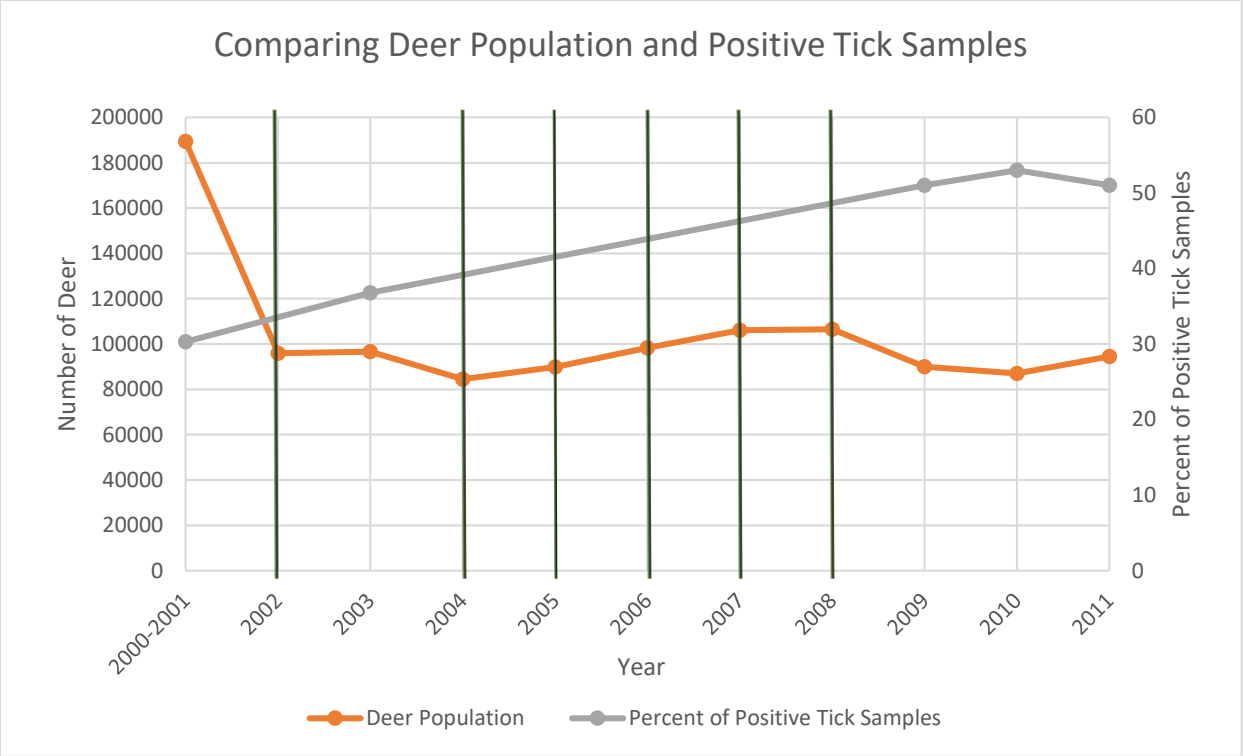

Figure 4 The above graph compares deer population data, which was acquired from NH Fish and Game, and positive tick samples. Deer data was estimated based on Maine population model with 80% confidence interval. The bolded, green vertical lines are to mark the lack of data available for rate of positive tick samples in the years 2002, 2004, 2005, 2006, 2007, and 2008.

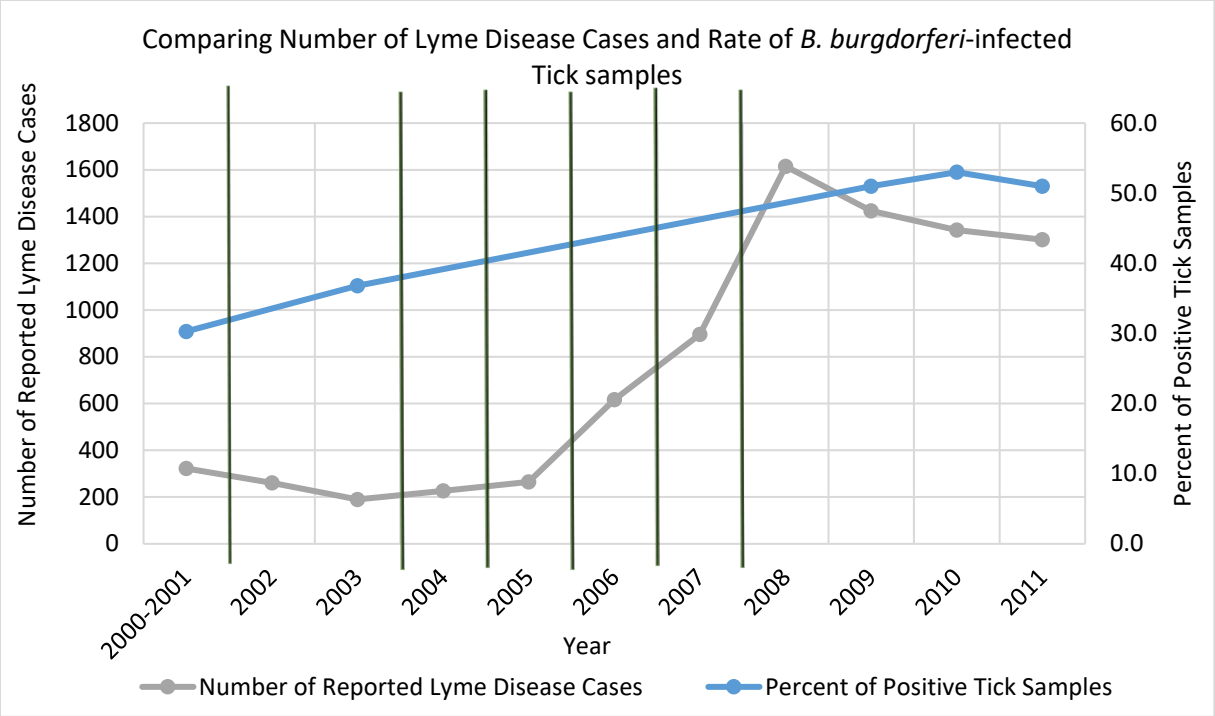

Figure 5 The above graph compares number of reported Lyme disease cases in NH, which was obtained from NH Department of Human and Health Services, and positive tick samples. The bolded, green vertical lines are to mark the lack of data available for rate of positive tick samples in the years 2002, 2004, 2005, 2006, 2007, and 2008.

DISCUSSION

DNA Amplification and Quantification

A study from 1995 reported successful PCR amplification of ticks, which had been preserved in 70% ethanol for 100 years (Hubbard et al. 1995). However, a 2014 study, that assessed methods of preserving *Rhipicephalus appendiculatus* (Acari: Ixodidae), reported unsuccessful PCR amplification of ticks preserved in 70% ethanol. The stark difference between the two studies can be attributed to the difference in size of amplified DNA. The 1995 study targeted part of the 16S mitochondrial rRNA gene, which yielded DNA fragments of 180 bp long. On the other hand, the 2014 study targeted two genes: ITS2 (250 bp) and cytochrome c oxidase subunit I (793 bp). Since the 2014 study's measurement for successful PCR amplification involve two genes—one of which is almost 800 bp long—it is unsurprising that the rate of success was lower than the 1995 study, which targeted a small-sized gene. Since both this study and the 1995 study targeted a small portion of highly-conserved genes, both studies reported successful amplification of historic, ethanol-preserved tick samples. Approximately 31% of the samples of sample group 2000, 2001, and 2003 were positive for the detection of *B. burgdorferi* by Real-Time TaqMan PCR. As a result, these tick samples are the oldest, documented samples that tested positive for *B. burgdorferi* in the state of New Hampshire. Prior to this study, the oldest, documented tick samples in the state of New Hampshire was from 2009.

It is also important to note that the positivity rates of the 2000, 2001, and 2003 tick samples could potentially be higher than the result of the study. The DNA concentrations of the negative tick samples were too low to be quantified by the Qubit Fluorometer, which can be attributed to significant degradation of DNA in the samples. Therefore, some of those tick samples may have been false negatives. Two potential explanations for the poor DNA

concentrations of the samples are the method of preservation and duration of storage. The tick samples from the years 2000 to 2003 had been preserved in 70% ethanol at room temperature, then stored at 4°C from 2016 until they were processed in 2017. Of note, some of the tick specimens were dry due to inadequate immersion of ticks in ethanol, which can be attributed to improper sealing of vacutainers to prevent fluid loss. A 2014 study reported that dried *R. appedndiculatus*, which had been stored at 4°C for an unspecified duration, had the lowest PCR success rate of 13.3%, while specimens preserved in 70% ethanol at 4°C for 10 years had the second lowest PCR success rate of 26.7% (Mtambo, et al. 2006). It is noteworthy that the aforementioned results of the 2014 study were comparable to this study. It is also possible that the duration of storage influenced the quality of DNA in the samples. A study in 2001, which analyzed the effect storage time on DNA quality of *Drosophila simulans*, reported that time of storage affected DNA yield and PCR success rate (Dean & Ballard 2001). In the 2001 study, specimens were preserved in either cyanide or 70% ethanol for two years. After the two-year storage period, both cyanide-preserved and ethanol-preserved specimens were noted to have sheared DNA due to endonuclease activity. The study attributed DNA degradation to prolonged storage, thus the samples had lower yield of DNA and success rate of PCR amplification than fresh specimens.

We were unsuccessful in our attempt to conduct next generation sequencing to further analyze tick samples. Initially, we intended to enriched the prokaryotic genome of tick samples. Following enrichment, we planned to process the enriched prokaryotic genome for next generation sequencing by Illumina. However, all of the tick samples had inadequate amounts of DNA for enrichment. Future studies should consider cryopreservation of tick samples at -80°C to preserve DNA for an extended period of storage. As seen in this study and another study,

cryopreservation of tick samples appears to be the most optimal method for DNA preservation (Mtambo et al. 2006). With adequate funding and resources, it is highly suggested for future studies to conduct amplicon sequencing to analyze *B. burgdorferi* at a genomic level. Through sequencing, we can investigate for any genetic variance within the *B. burgdorferi* genome, that may improve its adaptability to survive in its environment. Amplicon sequencing can also provide the opportunity to further investigate the presence of either antibiotic-resistance gene or efflux pump within *B. burdorferi*.

Correlation Between Tick Samples and the Environment

The rates of positive tick samples were correlated to both reported cases of Lyme disease and white-deer population. Due to lack of funding, tick collection and analysis were not done annually. Therefore, it is important to understand that the results obtained in this study are limited and may not accurately represent the relationship between ticks and the environment. However, the results of this limited study are suggestive of possible trends that need to be further analyzed and monitored, as they can be used for public health measures. Both the rates of positive samples and reported cases of Lyme disease are trending similarly over time, which further supports the spread of *B. burgdorferi*-infected ticks in NH. Based on the comparison between tick data and deer population, there was an indirect, inverse relationship between two datasets. Therefore, decreasing the white-tailed deer population may not decrease the spread of Lyme disease. A common public health measure to control tick population, which has taken place in Connecticut and Massachusetts, involves the control of white-tailed deer population. As previously mentioned, white-tailed deer are primary reproductive hosts for ticks and can affect the nymph population. However, based on this study and previous studies, white-tailed deer population cannot be directly correlated to Lyme disease incidence (Kugeler et al. 2015). Instead,

the indirect relationship between deer population and Lyme disease incidence may potentially signify the impact of tick host diversity on Lyme disease incidence. Previous studies have demonstrated that host species-rich communities typically report less Lyme disease incidence than host species-poor communities (LoGiudice et al. 2008; Turney et al. 2014). As previously discussed, white-footed mouse are natural reservoirs of *B. burgdorferi*. Ticks become infected with *B. burgdorferi* when they feed on white-footed mouse. Due to lack of competition, white-footed mouse is predominantly found in species-poor communities, consequently increasing the likelihood of ticks acquiring *B. burgdoferi* infection. In species-rich communities, white-footed mouse population no longer dominates the community, thus effectively decreasing the likelihood of ticks infected with *B. burgdorferi*, which decreases the Lyme disease incidence. Since reforestation in the late nineteenth century, species-diversity has increased due to additional resources available to support forest life (Turney et al. 2014). Therefore, it is possible that any change in the white-tailed deer population, whose habitat is forested areas, is indicative of a change in tick host diversity. It is highly suggested that future studies analyze the potential use of white-tailed deer population as a sentinel indicator for tick host diversity, which can be used as an early-risk assessment to estimate the abundance of *B. burgforeri*-infected ticks.

METHODS

Preservation of Tick Specimens

Sample group (2000, 2001, and 2003): Whole tick samples were collected by Dr. Alan Eaton, an entomologist from the University of New Hampshire. Upon collection, tick samples were placed in vacutainers with 70% ethanol at room temperature. In 2016, samples were sent to the State of New Hampshire Public Health Laboratories. Upon reception at the state laboratory, the tick samples were maintained in its media at 4°C until they were processed in 2017.

Sample group (2010): Tick samples were collected and homogenized by Maine Medical Center, contracted employees of State of New Hampshire Public Health Laboratories. Tick samples had been processed upon collection in 2010. Homogenized tick samples were preserved at -80°C until repeat processing in 2018.

Preparation of Tick Specimens

Ticks was sorted into its individual tube. 1,000 μL deionized water was added into each tube to rinse the ticks. Water was removed from the tubes, then 750 μL phosphate buffer solution (PBS) was added into each tube. Samples were stored at 4°C.

Genomic DNA Extraction

One stainless steel bead was added into each tube. Tubes were then placed in Mixer Mill at 20 Hz for 10 minutes. Liquid from each tube was moved into a new tube, then centrifuged at 13,000 rpm for 5 minutes. 200 μL supernatant from each sample was added to a well-plate.

The following procedure was done following Qiagen Cador Pathogen Kit (Qiagen, Hilden, Germany). Carrier RNA was resuspended in 310 μL Buffer AVE. The resuspended RNA was mixed with 2480 μL proteinase K. The mixture was combined with 9.9 mL VXL to create lysis buffer. 100 μL lysis buffer was mixed into each well, which contained tick specimen. The

samples were incubated at room temperature for 10 minutes. Well plate was placed into QIAcube HT instrument for DNA extraction. The DNA extracts were stored at -20°C.

Real-Time PCR

The Roche Lightcycler Faststart (Roche Life Science, Penzberg, Germany) was the kit used in this procedure. Primers and probe used in this study are as listed in Table 3. 12.0 µL deionized water was added into the well. 4 µL magnesium chloride and 2.5 µL 10x reaction mix were added into a reaction well. 0.5 µL of 20 µM forward primer, 0.5 µL of 20 µM reverse primer, and 0.5 µL of 20 µM probe were added into the reaction well as well. Reaction wells were then processed in ABI 7500 Fast DX. Thermocycling condition for PCR was conducted as detailed in Table 4.

Table 3 The following table lists the primers used for PCR amplification (Zeidner et al., 2001). FAM is the reporter dye on the probe. TAMRA is the quencher on the probe.

Type of Primer	Primer Sequence
Forward Primer	5'-TGGTGACAGAGTGTATGATAATGGAA-3'
Reverse Primer	5'-ACTCCTCCGGAAGCCACAA-3'
Probe	5'-FAM-TGCTAAAATGCTAGGAGATTGTCTGTCGCC TAMRA-3'

Table 4 The following table details the thermocycling condition for PCR with the Roche Lightcycler Faststart kit. The extension stage of PCR was not part of the NH Public Health Laboratories' state protocol. Due to the short amplicon size of 276 bp, the elongation of DNA strand occurs during the brief interval between denaturation and annealing phases.

	Temperature (°C)	**Duration**	**Number of Cycles**
Initial Denaturation	95	8 minutes	1
Denaturation	95	5 seconds	45
Annealing	60	30 seconds	

Quantification of DNA

The following procedure was done with Qubit Fluorometer (Thermofischer Scientific, Ipswich, MA) and according to manufacturer's instruction.

Concentrating and Pooling of DNA samples

The following procedure was conducted using Epigentek's DNA Concentrator Kit (Epigentek, Farmingdale, NY). 20 mL of 100% ethanol was mixed with CA2 buffer. 2 volumes of CA1 buffer were mixed with each volume of pooled DNA sample. The mixture was transferred to a silica column, then centrifuged at 12,000 rpm for 15 seconds. The flow-through was discarded. 200 µL CA2 was added to the column, then centrifuged at 12,000 rpm for 15 seconds. The flow-through was discarded and the washing step was repeated. 10 µL CA3 was added to the column, then centrifuged at 12,000 rpm for 20 seconds to elute DNA.

REFERENCES

Bouchard, C., Leighton, P. A., Beuchamp, G., Nguon, S., Trudel, L., Milord, F., . . . Ogden, N. H. (2013). Harvested White-Tailed Deer as Sentinel Hosts for Early Establishing Ixodes scapularis Populations and Risk from Vector-Borne Zoonoses in Southeastern Canada. *Journal of Medical Entomology*, 384-393.

Dean, M. D., & Ballard, J. W. (2001). Factors affecting mitochondrial DNA quality from museum preserved Drosophila simulans. *Entomologia Experimentalis et Applicata*, 279-283.

Deblinger, R. D., Wilson, M. L., Rimmer, D. W., & Spielman, A. (1993). Reduced abundance of immature Ixodes dommini (Acari: Ixodidae) following incremental removal of deer. *Journal of Medical Entomology*, 144-150.

Division of Public Health Services. (2015, March 31). *State of New Hampshire Tickborne Disease Prevention Plan.* Retrieved from Department of Human and Health Services: https://www.dhhs.nh.gov/dphs/cdcs/lyme/documents/tbdpreventionplan.pdf

Division of Public Health Services. (2016, June). *Publications and Data for Lyme and Other Tickborne Diseases.* Retrieved from NH Department of Health and Human Services: https://www.dhhs.nh.gov/dphs/cdcs/lyme/documents/tick-borne-bulletin.pdf

Hubbard, M. J., Cann, K. J., & Wright, D. J. (1995). Validation and rapid extraction of nucleic acids from alcohol-preserved ticks. *Experimental & Applied Acarology, 19*, 473-478.

Kugeler, K. J., Jordan, R. A., Schulze, T. L., Griffith, K. S., & Mead, P. S. (2015). Will Culling White-Tailed Deer Prevent Lyme Disease? *Zoonoses Public Health*, 337-345.

LoGiudice, K., Duerr, S. T., Newhouse, M. J., Schmidth, K. A., Killilea, M. E., & Ostfeld, R. S. (2008). Impact of Host Community Composition on Lyme DIsease Risk. *Ecology, 89*(10), 2841-2849.

Magnarelli, L. A., Anderson, J. F., & Cartter, M. L. (1993). Geographic distribution of white-tailed deer with ticks and antibodies to Borellia burgdorferi in Connecticut. *Yale Journal of Biology and Medicine*, 19-26.

Martin, N. (2010, August). *Effects of Climate Change on the Distribution of Whitefooted mouse (Peromyscus leucopus), an Ecologically and Epidemiologically Important Species.* Retrieved from University of Michigan Library: https://deepblue.lib.umich.edu/bitstream/handle/2027.42/78212/NadiaMartin_SNREthesis.pdf?sequence=1&isAllowed=y

Mtambo, J., Bortel, W. V., Madder, M., Roelants, P., & Backeljau, T. (2006). Comparison of preservation methods of Rhipicephalus appendiculatus (Acari: Ixodidae)for reliable DNA amplification by PCR. *Experimental and Applied Acarology*, 189-199. doi:10.1007/s10493-006-0004-4

Picken, R. N. (1992). Polymerase chain reaction primers and probes dervied from flagellin gene sequences for specific detection of the agents of Lyme disease and North American relapsing fever. *Journal of Clinical Microbiology, 30*(1), 99-114.

Turney, S., Gonzalez, A., & Millien, V. (2014). The negative relationship between mammal host diversity and Lyme disease incidence strengthens through time. *Ecology*, *95*(12), 3244-3250.

Werden, L., Barker, I. K., Bowman, J., K, G. E., Leighton, P. A., R, L. L., & Jardine, C. M. (2014). Geography, deer, and host biodiversity shape the pattern of Lyme disease

emergence in the Thousand Islands Archipelago of Ontario, Canada. *PLOS One*. doi:https://doi.org/10.1371/journal.pone.0085640

Wilson, M. L., Telford III, S. R., Piesman, J., & Spielman, A. (1988). Reduced abundance of immature Ixodes dammini following elimination of deer. *Journal of Medical Entomology*, 224-228.

Zeidner, N. S., Schneider, B. S., Dolan, M. C., & Piesman, J. (2001). An Analysis of Spirochete Load, Strain, and Pathology in a model of Tick-Transmitted Lyme Borrelosis. *Vector Borne and Zoonotic Diseases, 1*(1), 35-44. doi:10.1089/153036601750137642